

THE USE OF AN EPIOTOPE OF VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR KDR/FLK-1

The present invention relates to the use of the Y1214 epitope of the KDR/Flk-1 receptor as a marker in the measurement of a change in the activation state of the KDR/Flk-1
5 receptor. The invention also relates to the use of the KDR/Flk-1 epitope Y1214 as a marker in the detection of and/or measurement of the level of the KDR/Flk-1 receptor and to assays which utilise the use of the Y1214 epitope and to compounds derived from said assays.

KDR/Flk-1 is one of the receptors which binds vascular endothelial growth factor (VEGF), a growth factor which is engaged in the processes of angiogenesis. Three receptors
10 have been identified which bind VEGF, VEGFR-1 (also known as Flt-1), VEGFR-2 (also known as KDR or Flk-1) and VEGFR-3 (also known as Flt-4). For more information on the receptors bound by VEGF the reader is referred to the following review articles: Clauss, 2000 Seminars in Thrombosis and Hemostasis 26: 561-569; McMahon 2000, The Oncologist 5: 3-10; and Karkkainen & Petrova, 2000, Oncogene 19: 5598-5605.

15 Normal angiogenesis plays an important role in a variety of processes including embryonic development, wound healing and several components of female reproductive function. However, angiogenesis is also involved in a number of disease conditions. For example, undesirable or pathological angiogenesis has been associated with disease states including diabetic retinopathy, psoriasis, cancer, rheumatoid arthritis, atheroma, Kaposi's
20 sarcoma and haemangioma (Fan et al, 1995, Trends Pharmacol. Sci. 16: 57-66; Folkman, 1995, Nature Medicine 1: 27-31). VEGF also has effects on vascular permeability, which is thought to play a role in both normal and pathological physiological processes (Cullinan-Bove et al, 1993, Endocrinology 133: 829-837; Senger et al, 1993, Cancer and Metastasis Reviews, 12: 303-324).

25 The observation that angiogenesis is involved in a number of disease conditions has led to a number of approaches being undertaken to treat diseases by the modulation of angiogenesis. One of these approaches has been to develop inhibitors of the activity of receptors which bind VEGF. VEGF receptors belong to a family of receptors, called receptor tyrosine kinases (RTKs). RTKs are important in the transmission of biochemical signals across
30 the plasma membrane of cells. These transmembrane molecules characteristically consist of an extracellular ligand-binding domain connected through a segment in the plasma membrane to an intracellular tyrosine kinase domain. Binding of ligand to the receptor results in stimulation of the receptor-associated tyrosine kinase activity which leads to phosphorylation of tyrosine

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residues on both the receptor and other intracellular molecules. These changes in tyrosine phosphorylation initiate a signalling cascade leading to a variety of cellular responses. (For a review of RTKs, the reader is referred to: Schlessinger, 2000, Cell, 103:211-255. Examples of inhibitors of the VEGF receptor tyrosine kinase (VEGF RTK) can be found in the following

5 patent applications: International Application, Publication Number WO97/42187 and International Application, Publication Number: WO98/13350.

In addition to the need for inhibitors of the VEGF RTK, there is also a need for tools to characterise the activation and deactivation of the VEGF RTK. Such tools would be useful, for example, in studies characterising the biochemistry of the VEGF RTK and in the

10 measurement of changes in the activation state of the VEGF RTK during clinical studies of inhibitors of the receptor, thus giving an indication of the therapeutic efficacy of said inhibitors.

One approach to the development of such tools is to look for amino acid epitopes on the VEGF receptor which are phosphorylated during activation of the VEGF RTK. Such epitopes could be used, for example, in following the activation and de-activation states of the

15 VEGF RTK. Takahashi et al, 2001, EMBO Journal 20: 2768-2778 have identified two epitopes on the KDR/Flk-1 sub-type of the VEGF receptor, these are the tyrosine residues located at positions 1175 and 1214 in the amino acid sequence of the KDR/Flk-1 receptor (referred to hereafter as Y1175 and Y1214 respectively). In this paper the authors characterised these two epitopes and observed that Y1175 but not Y1214 was a major

20 phospholipase C-gamma (PLC γ) binding site linked to activation of MAP kinase, an important protein downstream of the biochemical pathways which is indirectly activated by binding of VEGF to the KDR/Flk-1 receptor. Thus, the authors concluded that Y1175 but not Y1214 plays a crucial role in the transduction of signals from the KDR/Flk-1 receptor to the MAP kinase pathway and DNA synthesis in endothelial cells. Thus, teaching that Y1175, but not

25 Y1214, would be an important marker for following activation of the KDR/Flk-1 receptor. We have raised antibodies against the Y1214 epitope and surprisingly found that we could use this epitope to follow activation of the KDR/Flk-1 receptor, observing inhibition of the phosphorylation of Y1214 by inhibitors of the KDR/Flk-1 RTK (as shown in Figures 1 below). We have also found that inhibitors of the KDR/Flk-1 RTK also inhibit phosphorylation of MAP

30 Kinase in parallel with inhibition of the phosphorylation of the Y1214 epitope. Thus, surprisingly we have found that Y1214 is an important marker for following the activation of the KDR/Flk-1 receptor.

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Thus, according to the first aspect of the present invention there is provided the use of the KDR/Flk-1 epitope Y1214 as a marker in the measurement of a change in the activation state of the KDR/Flk-1 receptor.

The Y1214 epitope could be used to detect the presence of the KDR/Flk-1 receptor in biological samples and to measure the levels of the KDR/Flk-1 receptor in biological samples. Thus, according to a further feature of the first aspect of the invention there is provided the use of the KDR/Flk-1 epitope Y1214 as a marker in the detection of and/or measurement of the level of the KDR/Flk-1 receptor.

One approach to allow such detection and/or measurement of the KDR/Flk-1 receptor is to develop probes to the Y1214 epitope, these could be developed to either the phosphorylated or de-phosphorylated epitope. Thus, according to a second aspect of the invention there is provided a probe directed to the KDR/Flk-1 epitope Y1214. Examples of such probes include binding proteins such as antibodies.

According to a further feature of the second aspect of the invention there is provided an antibody which specifically binds to epitope Y1214. A peptide fragment of the KDR/Flk-1 receptor comprising the Y1214 epitope may be utilized to prepare antibodies that specifically bind to Y1214. A specific example of such antibody preparation is described in Example 1 herein.

Polyclonal antibodies can be readily generated from a variety of sources, for example, horses, cows, goats, sheep, dogs, chickens, rabbits, mice or rats, using procedures that are well-known in the art. In general, a peptide comprising the Y1214 epitope is administered to the host animal typically through parenteral injection. The immunogenicity of a peptide comprising the Y1214 epitope may be enhanced through the use of an adjuvant, for example, Freund's complete or incomplete adjuvant. Following booster immunizations, small samples of serum are collected and tested for reactivity to the Y1214 epitope.

Examples of various assays useful for such determination include those described in: *Antibodies: A Laboratory Manual*, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988; as well as procedures such as countercurrent immuno-electrophoresis (CIEP), radioimmunoassay, radioimmunoprecipitation, enzyme-linked immuno-sorbent assays (ELISA), dot blot assays, and sandwich assays, see U.S. Patent Nos. 4,376,110 and 4,486,530.

Monoclonal antibodies may be readily prepared using well-known procedures, see for example, the procedures described in U.S. Patent Nos. RE 32,011, 4,902,614, 4,543,439 and 4,411,993; *Monoclonal Antibodies, Hybridomas: A New Dimension in Biological*

Analyses, Plenum Press, Kennett, McKearn, and Bechtol (eds.), (1980), each of which is incorporated herein by reference.

The monoclonal antibodies of the invention can be produced using alternative techniques, such as those described by Altling-Mees et al., "Monoclonal Antibody Expression
5 Libraries: A Rapid Alternative to Hybridomas", *Strategies in Molecular Biology* 3: 1-9 (1990) each of which is incorporated herein by reference. Similarly, binding partners can be constructed using recombinant DNA techniques to incorporate the variable regions of a gene that encodes a specific binding antibody. Such a technique is described in Larrick et al., *Biotechnology*, 7: 394 (1989), which is incorporated herein by reference. Monoclonal
10 antibodies can also be produced using cell lines, such as CHO cells or NS0 cells, or micro-organisms, such as bacteria or yeast, into which the genes for the heavy and light chains of the antibody have been transfected. Examples of such methods can be found in US 6331415, US 5876961, US 5545403 and US 5807715, each of which is incorporated by reference.

15 Thus, according to a further aspect of the invention there is provided a monoclonal antibody which specifically binds to the KDR/Flk-1 epitope Y1214.

According to a further aspect of the invention there is provided a hybridoma which produces an antibody which specifically binds to the KDR/Flk-1 epitope Y1214, preferably a monoclonal antibody.

20 According to a further aspect of the invention there is provided a recombinant cell line transfected with the cDNA for the expression of the heavy and light chains for an antibody which specifically binds to the KDR/Flk-1 epitope Y1214.

Once isolated and purified, the antibodies against Y1214 may be used to detect the presence of Y1214 in a sample using established assay protocols. In addition such antibodies
25 could be used to measure the levels of the KDR/Flk-1 receptor in samples. Further, the antibodies of the invention may be used therapeutically to bind to the Y1214 epitope and inhibit its activity *in-vivo*.

Thus, according to a further aspect of the invention there is provided the use of an antibody which binds to the KDR/Flk-1 epitope Y1214 in the manufacture of a medicament,
30 preferably a medicament for the treatment of cancer.

According to a further aspect of the invention there is provided a method of treatment using an antibody which binds to the KDR/Flk-1 epitope Y1214, preferably a method for the treatment of cancer.

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Another approach to the use of the KDR/Flk-1 epitope Y1214 as a marker in the measurement of a change in the activation state of the KDR/Flk-1 receptor is to measure the phosphorylation state of the KDR/Flk-1 receptor activated in the presence of adenosine triphosphate labelled with radioactive phosphate (for example, labelled with phosphorous 32) and to determine the level of radioactivity of the KDR/Flk-1 receptor, preferably the level of radioactivity in peptide fragments of the KDR/Flk-1 receptor comprising Y1214.

A further approach to the use of the KDR/Flk-1 epitope Y1214 as a marker in the measurement of a change in the activation state of the KDR/Flk-1 receptor is to use nuclear magnetic resonance (NMR) to follow changes in the phosphorylation state of Y1214.

According to a further aspect of the invention there is provided a method of generating a probe directed to the Y1214 epitope of the KDR/Flk-1 receptor. Preferably the generation of an antibody.

According to a further aspect of the invention there is provided a method of generating an antibody, which comprises:

- (i) immunizing a mammal with a peptide which comprises the KDR/Flk-1 epitope Y1214; and
- (ii) isolating an antibody from said mammal.

The skilled man would have access to a large number of assay techniques with which to develop assays to detect, measure the levels of, and/or measure a change in the activation state of the KDR/Flk-1 receptor, based on the knowledge of the importance of the Y1214 epitope. Examples of such assay techniques include, fluorimetric assays, chromogenic assays, radiolabelled assays or chemiluminescence assays. Thus, according to a third aspect of the invention there is provided an assay method to detect, measure the levels of, and/or measure a change in the activation state of the KDR/Flk-1 receptor utilising Y1214 as a marker, preferably to measure a change in the activation state of the KDR/Flk-1 receptor.

For guidance for the design of biological assays the skilled man will be aware of a number of standard works. Examples of such standard works include: Knight (1995) Fluorimetric Assays of Proteolytic Enzymes, Methods in Enzymology 248, 18-34; Chard (1978) An Introduction to Radioimmunoassay and related techniques, Elsevier/North-Holland Biomedical Press; and Basic Methods in Molecular Biology. First author Leonard G Davis. 1986 Elsevier Science Publishing Co. Inc., Amsterdam.

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According to a further feature of the third aspect of the invention there is provided an assay method for the measurement of a change in the activation state of the KDR/Flk-1 receptor, comprising

- (a) mixing a biological sample with an assay mixture comprising one or more probes
5 directed to the KDR/Flk-1 epitope, Y1214 ; and
- (b) measuring a signal which is proportional to the proportion of the KDR/Flk-1 epitope, Y1214 which is in the phosphorylated or un-phosphorylated state.

According to a further feature of the third aspect of the invention there is provided an assay method for the measurement of a change in the activation state of the KDR/Flk-1
10 receptor, comprising

- (a) mixing a biological sample with an assay mixture comprising one or more probes directed to the KDR/Flk-1 epitope, Y1214 ; and
- (b) measuring the activation state of the KDR/Flk-1 receptor in the biological sample.

According to a fourth aspect of the invention there is provided a diagnostic kit to
15 detect, measure the levels of, and/or measure a change in the activation state of the KDR/Flk-1 receptor comprising reagents for preparing an assay mixture comprising a probe of the invention and instructions for use thereof. Preferably said diagnostic kit is directed towards measuring the activation state of the KDR/Flk-1 receptor.

According to a further feature of the fourth aspect of the invention there is provided a
20 diagnostic kit to detect, measure the levels of, and/or measure a change in the activation state of the KDR/Flk-1 receptor in a biological sample, for use with a method of the invention, the diagnostic kit comprising reagents for preparing an assay mixture and instructions for use thereof.

According to a further feature of the fourth aspect of the invention there is provided a
25 method of determining the effective dose of an inhibitor of the KDR/Flk-1 receptor, which comprises:

- (a) dosing one or more humans or other mammals with a range of KDR/Flk-1 receptor inhibitor concentrations, preferably one or more humans;
- (b) isolating a biological sample from said humans or other mammals, preferably a cell
30 sample;
- (c) measuring a signal proportional to the activation state of the KDR/Flk-1 receptor using an assay method of the invention; and
- (d) calculating the effective dose of the inhibitor from the measured signal.

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It would be clear to the skilled man that the KDR/Flk-1 receptor inhibitor is an inhibitor of the activation of the KDR/Flk-1 receptor by binding of a ligand, such as VEGF, to the receptor. This could occur at the VEGF binding site or at an allosteric site. Such inhibitors include inhibitors of the KDR/Flk-1 receptor tyrosine kinase or agents which blocks binding of ligands to the receptor, for example antibodies directed to the KDR/Flk-1 receptor. A preferred inhibitor is *N*-(4-bromo-2-fluorophenyl)-6-methoxy-7-[(4-methylpiperidin-1-yl)methoxy]quinazolin-4-amine.

According to a further feature of the fourth aspect of the invention there is provided a method of determining the effective therapeutic dose of a KDR/Flk-1 receptor inhibitor which comprises:

- (a) dosing one or more humans or other mammals with a range of KDR/Flk-1 receptor inhibitor concentrations, preferably one or more humans;
- (b) isolating a biological sample from said humans or other mammals, preferably a cell sample;
- (c) measuring the activation state of the KDR/Flk-1 receptor using a method of the invention; and
- (d) calculating the effective therapeutic dose of the inhibitor from the measured activation state.

According to a the fifth aspect of the invention there is provided a method of preparing a pharmaceutical composition of a KDR/Flk-1 receptor inhibitor which comprises:

- (a) determining the effective dose of the inhibitor according to a method of the invention; and
- (b) preparing a unit dose of inhibitor comprising an amount of inhibitor within the effective dose range and a pharmaceutically acceptable excipient.

According to a further feature of the fifth aspect of the invention there is provided a method of preparing a pharmaceutical composition which comprises:

- (a) determining the effective therapeutic dose of a KDR/Flk-1 receptor inhibitor by a method of the invention, and
- (b) mixing the inhibitor in an amount within the effective therapeutic dose range with a pharmaceutically acceptable excipient.

According to a further feature of the fifth aspect of the invention there is provided a pharmaceutical composition prepared by one of the above methods of preparing a pharmaceutical composition.

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According to a sixth aspect of the invention there is provided a method of determining whether a chemical compound is an *in-vivo* inhibitor of the KDR/Flk-1 receptor tyrosine kinase activity which comprises, measuring the degree of phosphorylation of Y1214 in a biological sample obtained from a subject to whom said chemical compound has been administered.

According to a seventh aspect of the invention there is provided the use of the degree of phosphorylation of Y1214 as a surrogate marker of KDR/Flk-1 receptor tyrosine kinase inhibitory activity of a chemical compound.

The skilled man will appreciate that this effective therapeutic dose may be formulated in one or more preparations to be delivered to the patient over a defined period of time. The skilled man will also appreciate that once the effective dose has been determined further pharmaceutical compositions can be prepared which will have been indirectly prepared by the above methods.

Within this specification the following terms are defined as follows:

- 15 **"Activation state"** - any change in the molecular conformation of the KDR/Flk-1 receptor which leads to the direct or indirect modulation of the activity of another protein or other proteins within the cell membrane or any other part of a animal or human cell. Preferably the change in conformation is induced directly or indirectly by the phosphorylation of one or more amino acid residues within the KDR/Flk-1 receptor.
- 20 **"Antibodies"** include polyclonal antibodies, monoclonal antibodies, and the various types of antibody constructs such as for example F(ab')₂, Fab and single chain Fv. Antibodies are defined to be specifically binding if they bind Y1214 with a K_d of greater than or equal to about 10⁷ M⁻¹. Affinity of binding can be determined using conventional techniques, for example those described by Scatchard et al., *Ann. N.Y. Acad. Sci.*, 51:660 (1949).
- 25 **"Assay Mixture"** - comprises reagents suitable for the detection, measurement of the levels of, and/or measurement of a change in the activation state of the KDR/Flk-1 receptor; such reagents are readily selectable by a person skilled in the art. For example, a preferred assay mixture comprises a suitable buffer, one or more labelled probes directed to the KDR/Flk-1 epitope, Y1214, and optionally any other co-factors which may be required.
- 30 **"Biological sample"** is a sample of material derived from any component of an animal body. Such material includes, but is not limited to, blood, urine, tissue sections, and tissue biopsies, preferably tissue biopsies or blood. Tissue biopsies may be assayed as sections or may be assayed as a cell suspension. Biological samples may be derived from a variety of animal

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species, preferably mammals, more preferably human, monkey, dog, guinea pig, rat or mouse, further preferably rat, mouse or human, most preferably human. The skilled man would appreciate that a biological sample may be purified or partially purified before use.

'Biological sample' also includes cultured cell lines, and material derived therefrom. A

5 preferred biological sample is a needle biopsy from a tumour.

"Effective Dose" - for the avoidance of doubt effective dose means the effective therapeutic dose for the treatment of a disease wherein inhibition of the activity of the KDR/Flk-1 receptor would be of therapeutic benefit. The skilled man would be able to calculate the effective therapeutic dose from the degree of inhibition of the activity of the KDR/Flk-1

10 receptor in the biological sample.

"KDR/Flk-1 epitope Y1214" - for the avoidance of doubt, references to the KDR/Flk-1 epitope Y1214 relate to the epitope in both its un-phosphorylated and phosphorylated form.

"Modulation" - for the avoidance of doubt modulation can relate to either an increase in activity or a decrease in activity.

15 **"Probe"** - relates to any moiety which specifically binds to an amino acid sequence, for example a sequence containing the Y1214 epitope of the KDR/Flk-1 receptor.

"Signal" - for the avoidance of doubt a signal could be derived from a single probe or could be a number of signal derived from two or more probes. For example, a probe directed to the phosphorylated epitope could be used in combination with a probe directed to the

20 un-phosphorylated epitope and the resulting 2 signals be used to measure the proportion of receptor in the phosphorylated and un-phosphorylated state.

"Surrogate marker" - relates to any measurement of a biological or biochemical activity which indirectly indicates the efficacy of a chemical compound on the treatment of a disease state in an animal, preferably a human.

25 **"Y1214"** - relates to the tyrosine residue located at position 1214 in the amino acid sequence of the mature KDR/Flk-1 receptor. The sequence is set out in Terman et al 1991, Biochem Biophys Research Commun 187(3), 1579-1586. In the Terman et al sequence the initiator methionine is present, thus Y1214 is shown at position 1215 of the sequence. This methionine would be absent in the transcribed protein; thus the tyrosine residue relating to the present
30 invention would be present at position 1214. The sequence of KDR/Flk-1 can also be found at SwissProt Accession No. P35968.

The invention will now be illustrated with reference to the following non-limiting examples and accompanying figure, wherein:

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Figure 1: shows a dose dependent decrease in phosphorylation of the Y1214 epitope in response to increasing concentration of the KDR receptor tyrosine kinase inhibitor 4-[(4-fluoro-2-methyl-1*H*-indol-5-yl)oxy]-6-methoxy-7-(3-piperidin-1-ylpropoxy)quinazoline; where lane O = untreated HUVEC cell lysate, lane V = VEGF-treated HUVEC cell lysate and the remaining lanes show the effect of 0.007-5.0uM compound on VEGF-treated cells. The arrow shows the position of KDR on the membrane.

and the following abbreviations are used:

PBST – 0.05% Tween-20 in PBS.

BSA – bovine serum albumin;

HUVECs - human umbilical vein endothelial cells

PBS – phosphate-buffered saline; and

OVA – ovalbumin

Example 1 – Preparation of polyclonal antibodies directed to the KDR/Flk-1 epitope, Y1214

Conjugation of KDR/Flk-1 peptide to OVA using Maleimide

KDR/Flk-1 peptide “Ac-CDPKFHY(p)DNTAGIS-NH₂” was dissolved in PBS (0.1M pH=7.4) at a concentration of 1mg/ml and freeze-dried maleimide activated ovalbumin (ex Pierce & Warriner Cat. No. 77126) was dissolved in PBS at 1ml/mg. 3mls of peptide was then mixed with 3mls of maleimide-activated OVA and left to react for 4 hours at room temperature in the dark, with continuous mixing on a roller. After 4 hours the reaction mixtures were dialysed against 10 litres of stirred PBS overnight at 4°C (10000 molecular weight cut off). After dialysis the volumes of the reaction mixtures were adjusted to give final protein concentration of 1mg/ml. 200µl aliquots were then stored at -20°C.

Immunisation of rabbits with KDR/Flk-1 peptide conjugate

Three female New Zealand White rabbits were immunised with the ovalbumin conjugate of the KDR/Flk-1 peptide. Immunisations were carried out using the subcutaneous route, each dose being delivered to 4 sites on the back of the rabbit, in a total volume of 1ml. Four doses were given at four weekly intervals, with each dose containing 50µgs of conjugate, as follows.

Dose 1 - delivered as an emulsion, made up using a 1:1 mixture of PBS and Freund's Complete Adjuvant;

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Dose 2 - delivered as an emulsion, made up using a 1:1 mixture of PBS and Freund's

Incomplete Adjuvant;

Dose 3 - delivered as an emulsion, made up using a 1:1 mixture of PBS and Freund's

Incomplete Adjuvant; and

5 Dose 4 - delivered in PBS alone.

Pre-bleeds were taken prior to the first dose and 10 days after each subsequent dosing. The blood was removed from the marginal ear vein of the animal, allowed to clot for several hours at room temperature and then stored at 4°C overnight. The samples were then spun in a bench-top centrifuge for 15-30 minutes and the serum decanted from the pellet. The serum
10 samples were then frozen and stored at -20°C.

Assay of Test bleeds from Rabbits immunised with Phosphorylated peptide

Phosphorylated [Ac-CDPKFHY(p)DNTAGIS-NH₂]

Non Phosphorylated [Ac-CDPKFHYDNTAGIS-NH₂]

- 15 Assays were performed on 96 well polystyrene plates. Plates were coated with either phosphorylated or non-phosphorylated peptide at 2 µg/ml in bicarbonate buffer (0.1M, pH 9.6) and incubated for 8 hrs at room temperature. Plates were then washed once with PBS/Tween 20 (0.5%)(pH=7.4) and blocked with a 1% solution of dried, skimmed milk powder in PBS at 120 µl/well overnight at 4°C.
- 20 Test bleeds were then assayed as follows. Plates were then washed once with PBS/Tween20 (0.5%)(pH7.4), samples added to wells at 100 µl/well; doubling dilutions, starting with neat serum, in duplicate and then incubated for 3 hours room temperature. Plates were then washed three times with PBS/Tween20 (0.5%)(pH7.4) and a peroxidase labelled Goat anti Rabbit antibody (Sigma Chemical Co, Poole, Dorset, UK Catalogue Number: A-0545) diluted
25 to 1 in 4000 with PBS/Tween20 (0.5%)(pH7.4) was added at 100 µl/well. Plates were then incubated for 3 hours at room temperature. Plates were then washed four times with PBS/Tween20 (0.5%)(pH7.4). Then, peroxidase substrate, 600 µg/ml ortho-phenylene-diamine + 0.02% urea H₂O₂ (in citrate phosphate pH 5) substrate was added at 100 µl/well to the plates and incubated at room temperature. The reaction was stopped after 15 minutes by
30 the addition of 0.1M citric acid at 50 µl/well.
- Plates then read at 450nm using a spectrophotometric plate reader.

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Preparation of phosphorylated KDR/Flk-1 peptide and non-phosphorylated KDR/Flk-1 peptide affinity columns using "Sulfolink™"

Sulfolink™ is a 6% cross-linked beaded agarose gel with immobilised iodoacetyl groups for immobilising sulfhydryl-containing peptides. (Source: Pierce Chemical Company, Rockford,

5 Illinois, USA).

10mls of "Sulfolink" slurry was added to a disposable 5ml (bed volume) polypropylene columns with additional reservoir fitted at room temperature and allowed to sediment for 2 hours. The column was then washed twice with 25mls of 50mM Tris + 5mM Na-EDTA pH 8.5. Peptides were dissolved at 1mg in 5mls of Tris/EDTA (as above).

- 10 Affinity columns were prepared by adding a 5ml solution of either the phosphorylated or non-phosphorylated peptide, the columns were then mixed well using a long needle, capped and mixed on shaker for 15 minutes. After 15 minutes columns were set upright and left to settle for 30 minutes. Then columns were drained and 6mls of 50 mM Cysteine in Tris/EDTA run into each column. The columns were then capped, mixed thoroughly with
- 15 needle, and mixed for 15 minutes. Column were then set upright and allowed to settle for 30 minutes. 40mls of 1M NaCl was then run through each column, followed by 30ml 0.05% Sodium Azide . Columns were then stored at 4°C.

Purification of antibodies using non-phosphorylated peptide and phosphorylated peptide

20 affinity columns

Prior to column chromatography serum samples were purified by precipitation using 50% ammonium sulphate. The precipitates were resuspended in approximately 10mls of PBS and dialysed against 10 Litres of PBS overnight at 4°C (10,000 molecular weight cut off). The ammonium sulphate-purified samples were then further purified by chromatography on a

25 phosphorylated peptide affinity column followed by chromatography on a non-phosphorylated peptide column. Prior to use affinity columns prepared as described above with phosphorylated or non-phosphorylated peptide were brought to room temperature and washed with 20mls of PBS.

- 30 Column chromatography was performed as follows.

(i) phosphorylated peptide affinity column

10mls of ammonium sulphate purified material was made up to 50mls with PBS and added to the top of a phosphorylated peptide affinity column and allowed to run through, then the

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column was washed with 15mls of PBS. The column was then eluted with approximately 10mls of 100mM Glycine pH 3.0 and 10 x 1ml fractions were collected, with 100µl of 1M Tris pH 8.9 being added to collection vials from fraction 4-10. The elution from the column was checked by measuring the optical density of the fraction at 280nm in a spectrophotometer. The column was then regenerated by the addition of 10ml 100mM Glycine pH 2.5, followed by 20mls PBS and the column was stored at 4°C.

(ii) non-phosphorylated peptide affinity column

Fractions 3-8 were pooled and added to the non-phosphorylated peptide column, followed by 10mls of PBS. All material coming through the column was collected in 1ml fractions and the optical density at 280nm measured checked on a spectrophotometer. Fractions 2 - 7 were then pooled and dialysed against PBS, overnight at 4°C. After dialysis, the samples were stored at 4°C for immediate use, or at -20°C for storage.

Assay of Rabbit Fractions from phosphorylated peptide and non-phosphorylated peptide affinity columns

96 well plates were coated for 4 hours at room temperature with 2µg/ml of peptide in 0.1M bicarbonate buffer pH 9.6. The plates were then washed once with PBS/Tween20 (0.5%)(pH=7.5) and blocked with a 1% solution of dried, skimmed milk powder at 200µl/well for 1 hour at room temperature. Plates were then washed once with PBS/Tween20 (0.5%)(pH7.5) and samples added to wells at 100µl/well. Fractions were assayed in doubling dilutions, starting at 25ug/ml of protein (by optical density at 280nm). Plates were then incubated at 4°C overnight.

Next morning plates were washed three times with PBS/Tween20 (0.5%)(pH7.5) and a goat anti-rabbit peroxidase antibody (Sigma Chemical Co, Poole, Dorset, UK, Catalogue Number:A-0545) diluted to 1 in 2000 with PBS/Tween20 (0.5%)(pH7.5) was added at 100µl/well. Plates were then incubated for 4 hours at 4°C. After 4 hours plates were washed four times with PBS/Tween20 (0.5%)(pH7.5). Then, the peroxidase substrate ortho-phenylene diamine + urea H₂O₂ (in citrate phosphate buffer pH=5) at 100ul/well was added. After 3 minutes the reaction was stopped by addition of 0.1M Citric acid 100ul/well. Plates were then read at 450nm on a spectrophotometric plate reader.

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Example 2 – Treatment of Human umbilical vein endothelial cells (HUVECs) with inhibitors of KDR/Flk-1 receptor tyrosine kinase

HUVECs were obtained from TCS Cellworks, Buckingham, UK (Catalogue number ZHS 8965) and cultured in Large Vessel Endothelial Cell Basal Medium + recommended
5 supplements obtained from TCS Cellworks (catalogue numbers ZHM 2951 and ZHS 8965). Cells were used when close to confluence in 3x75cm² tissue culture flasks. Prior to treatment with inhibitor cells were cultured overnight in Basal Medium without supplements.

HUVECS were treated with inhibitors as follows. Inhibitors were diluted in Basal Medium,
10 added to cultures at a final concentration of 1µM. and the cells incubated at 37°C for 82 minutes. After 82 minutes 50ng/ml VEGF was added to the cells and the cells incubated for a further 8 minutes at 37°C. After this further incubation the culture medium was removed from the cells and the cells lysed with 2.5ml sample buffer [Sample buffer: 4 parts NuPage sample buffer (Novex, NP0007, Invitrogen, Paisley, Scotland) to 1 part distilled water]. The lysate
15 was collected, 3µl of β-mercaptoethanol added and heated to 95°C for 10 minutes to ensure complete reduction and denaturation. The lysates can be stored at -20°C until analysed.

Example 3 – SDS PAGE of lysates

Lysates were analysed using SDS polyacrylamide gel electrophoresis (PAGE) to separate the
20 proteins within the lysates. Electrophoresis was performed using a 4-12% NuPage bis-tris gel (Invitrogen, Paisley, Scotland, UK, catalogue no. NP0321) and NuPAGE MOPS SDS Running Buffer (Invitrogen, Paisley, Scotland, UK, catalogue no. NP0001). After the electrophoresis the protein were transferred from the gels to nitrocellulose membrane using Novex blotting modules as per the manufacturers instructions (Invitrogen, Paisley, Scotland, UK, catalogue no.
25 LC2001). The membranes were then stained with Ponceau S (Sigma, Poole, Dorset, UK. Catalogue No. P-7170) and store air-dried, in sealed bags at 4°C.

Example 4 – Treatment of nitrocellulose membranes with anti-KDR/Flk-1 antibodies

Nitrocellulose membranes were incubated for 1 hour in 5% solution of dried, skimmed milk
30 powder in PBST [Marvel / PBST] at room temperature. After 1 hour the membrane was incubated for between 2-3 hours at room temperature with an anti-KDR/Flk-1 antibody diluted 1:100 in Marvel / PBST. After this period the membrane was washed four times for at least 5 minutes with PBST and then the membrane was incubated with an appropriate secondary

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antibody (e.g. NEB an anti-rabbit antibody bound to horse-radish peroxidase; used at a dilution of 1:2000) for 1 hour at room temperature. The membrane was then washed for 30 minutes using at least 4 changes of PBST, incubated with Lumiglo reagents (Cell Signaling Technology Inc, Cummings Center, Beverly, MA. USA) as directed. The membranes were then exposed to

5 X-ray film and the film developed to reveal the bands reactive to the anti-KDR/Flk-1 antibody. Lumiglo is a proprietary mixture of reagents containing a substrate which is converted to a chemiluminescent compound by the action of horseradish peroxidase. The light from this is detected by photographic film, for example Kodak Biomax ML.